BRIEF REPORT

Severe Acute Respiratory Syndrome–Related Coronavirus Is Inhibited by Interferon-α

Ute Ströber,[1,2] Antonino DiCaro,[4] Yan Li,[1,2] James E. Strong,[1] Fred Aoki,[2] Frank Plummer,[1,2] Steven M. Jones,[1,3] and Heinz Feldmann[1,2]

[1]National Microbiology Laboratory, Health Canada, and Departments of [2]Medical Microbiology and [3]Immunology, University of Manitoba, Winnipeg, Canada; [4]Laboratorio di Virologia–Padiglione Baglivi, Istituto Nazionale per le Malattie Infettive "L. Spallanzani"–IRCCS, Rome, Italy

Current treatment schemes for severe acute respiratory syndrome (SARS) include broad-spectrum antibiotics, glucocorticoids, and ribavirin. We evaluated the susceptibility of the SARS-related coronavirus (SARS CoV) to ribavirin and interferon (IFN)–α in vitro by use of cytopathic effect, plaque assay, and immunoblot analysis. Ribavirin did not inhibit viral growth at concentrations attainable in human serum. In contrast, IFN-α showed an in vitro inhibitory effect starting at concentrations of 1000 IU/mL. In conclusion, ribavirin alone is unlikely to be beneficial in the prophylaxis or treatment of SARS CoV infections. Clinical trials with IFN-α might be justified to determine a beneficial effect on the outcome of SARS.

As of 23 June 2003, the rapid worldwide dissemination of severe acute respiratory syndrome (SARS) has resulted in 30 countries reporting 8459 cases. The Asia Pacific region, including mainland China, has been most severely affected by the disease, but western countries, most notably Canada, also have reported cases. The global interest in the epidemic has pushed science forward, so that, within weeks of the outbreak being identified, the potential causative agent was isolated from several patients with SARS, and the full-length sequence was determined. Genetic analysis identified the agent as a newly emerged coronavirus (CoV) (order *Nidovirales*, family *Coronaviridae*, genus *Coronavirus*), which is different from all previously known groups of CoVs [1, 2].

Despite the early success in molecular characterization, progress has been less rapid in other areas, particularly in establishing an antiviral treatment for patients with SARS. SARS is primarily diagnosed by a process of clinical/epidemiological exclusion. Despite improvement in the recent past, laboratory diagnosis, particularly molecular detection of the virus by polymerase chain reaction, remains unreliable, especially in the first few days of the disease; in addition, the tests are not yet validated. Serologic analysis is thought to be the confirmatory laboratory test, but most patients with SARS develop detectable IgG antibody levels 3–4 weeks after the onset of symptoms. Thus, differential diagnosis remains a problem for the clinician at the time of initial presentation, particularly in individuals without known exposure to other patients with SARS or history of travel to a region where SARS is endemic.

The case-fatality rate of 9.5% reflects, in part, the lack of an effective specific treatment for this viral infection. Broad-spectrum antibiotics to treat community-acquired bacterial pneumonia, glucocorticoids, and ribavirin have been administered to patients with SARS, but their efficacy is unknown [3–5]. Recently, glycyrrhizin has been demonstrated to inhibit the SARS-related CoV (SARS CoV) in vitro [6]. Ribavirin has been used on the basis of its ability to inhibit other CoVs [7] and also showed an inhibitory effect on several other RNA viruses, such as bunyaviruses and arenaviruses [8]. To support the search for effective antiviral treatments, we evaluated the susceptibility of SARS CoV isolates (detailed studies were performed with the Tor2 isolate [Toronto, Canada]) to ribavirin and interferon (IFN)–α–2b in vitro. Our data indicate that ribavirin does not inhibit the virus at concentrations attainable in human serum but that IFN-α–2b may be useful and deserves further evaluation as a therapeutic agent.

Materials and methods. Studies were performed with the recently sequenced Tor2 isolate, obtained from a Toronto patient [1, 4]. The general findings were confirmed by use of 3 additional independently obtained isolates from Toronto patients (Tor3, Tor7, and Tor684). All isolates originated from nasopharyngeal swabs. Vero E6 cells (ATCC 1568) were used for the susceptibility studies. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco BRL).

Vero E6 cells were infected at an MOI of 0.001 with the SARS CoV. After 1 h of adsorption, the supernatants were replaced with DMEM containing various amounts of ribavirin (0–2000 μg/mL) or IFN-α–2b (0–5000 IU/mL) (Schering-Plough) for 72 or 96 h.

For immunoblot analysis, cell lysates were analyzed by SDS-PAGE on 10% Tris gels. Protein was electrotransferred to polyvinyl-

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Financial support: Health Canada severe acute respiratory syndrome–related fund.
Reprints or correspondence: Dr. Heinz Feldmann, 1015 Arlington St., Winnipeg, Manitoba, Canada R3E 3R2 (heinz_feldmann@hc-sc.gc.ca).

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idene difluoride membrane (Immobilon P membrane; Millipore). Viral antigen was detected by use of enhanced chemiluminescence, using a patient serum sample and horseradish peroxidase–conjugated anti–human IgG.

For plaque assay, confluent Vero E6 cells were infected in 10-fold dilutions of SARS CoV; 45 min after infection, the inoculum was removed, and cell monolayers were overlaid with 0.9% low-melting-point agarose in DMEM, with or without IFN-α–2b. Titers were determined 72 h after infection, following crystal violet staining.

For microassay, Vero E6 cells were infected with SARS CoV at an MOI of 0.001 for 45 min at 37°C and then were incubated in DMEM containing various amounts of IFN-α–2b (0–5000 IU/mL); 72 h after infection, cells were formalin fixed, removed from biocontainment, and analyzed by use of phase-contrast microscopy, using an Axiovert 200 microscope (Zeiss).

Results. In vitro studies were conducted in Vero E6 cells, which is one of only a limited number of cell lines susceptible to SARS CoV. Vero E6 cell monolayers were treated with ribavirin at various concentrations (0, 20, 50, 100, 200, 1000, and 2000 μg/mL) for 96 h. Treatment was initiated concurrently with inoculation of the cell cultures with virus at an MOI of 0.001. Inhibition of viral cytopathic effect (CPE) was used to determine the antiviral effect of the drug. No reduction in CPE was detected after 4 days, compared with untreated control samples. Viral titers determined from tissue culture supernatant were identical for treated and untreated cells and amounted to $\sim 10^6$ pfu/mL (data not shown). Ribavirin was tested up to concentrations 10 times greater (2000 μg/mL) than those that inhibit the replication of ribavirin-sensitive viruses, such as arenaviruses and bunyaviruses [9, 10]. There was no evidence that the SARS CoV was susceptible to the action of ribavirin.

IFN susceptibility has been demonstrated for several coronaviruses, such as mouse hepatitis virus, porcine transmissible gastroenteritis virus, feline infectious peritonitis virus, and human coronavirus [11–14]. Inhibitory concentrations ranged from 2 IU/mL [12] to 1000 IU/mL [14]. Therefore, we tested the susceptibility of the Tor2 isolate to IFN-α–2b (Schering-Plough). In brief, Vero E6 cells were infected at an MOI of 0.001, either 20 h after or at the time of treatment with IFN-α–2b (250–5000 IU/mL). Treatment was continued for 72 h. The CPE was used to demonstrate an antiviral effect. As indicated in figure 1, the CPE on day 3 after infection was significantly decreased in Tor2-infected cultures treated with 1000 IU/mL of IFN-α–2b in both treatment groups, compared with that in the untreated control group. This indicated that pretreatment was not necessary for the protective effect in tissue culture (data not shown). Almost complete protection was achieved when infected Vero E6 cells were treated with 5000 IU/mL of IFN-α–2b.

To quantify the effect of IFN-α–2b on the replication of the SARS CoV, Vero E6 cells were infected at an MOI of 0.001 and were incubated in the presence IFN-α–2b (0–5000 IU/mL), as described above. After 72 h, cells and supernatants were harvested to determine protein expression by immunoblot analysis and virus yield by plaque titration on Vero E6 cells. As indicated in figure 2*A*, a concentration of 1000 IU/mL of IFN-α–2b substantially reduced virus yields, and ~1 log decrease in virus yield was achieved at a dose of 2000 IU/mL of IFN-α–2b. The reduction in virus yield seems to be related to a decrease in virus growth, as supported by the change in the plaque morphology (figure 2*B*). Inhibition of viral protein expression was investigated by use of immunoblot analysis after SDS-PAGE of the cell lysates (figure 2*C*). A serum sample from a patient with SARS who seroconverted to the SARS CoV was used to detect viral proteins. Earlier studies had shown that this serum sample reacted strongly with the nucleocapsid protein of the SARS CoV [15]. Viral protein expression was reduced at a concentration of 1000 IU/mL and abolished at concentrations of 2000 and 5000 IU/mL of IFN-α–2b.

For confirmation, the effect of IFN-α–2b on 3 additional independent SARS CoV isolates (Tor3, Tor7, and Tor684) obtained from Canadian patients with SARS was determined. All isolates showed the same pattern of inhibition by IFN-α–2b as

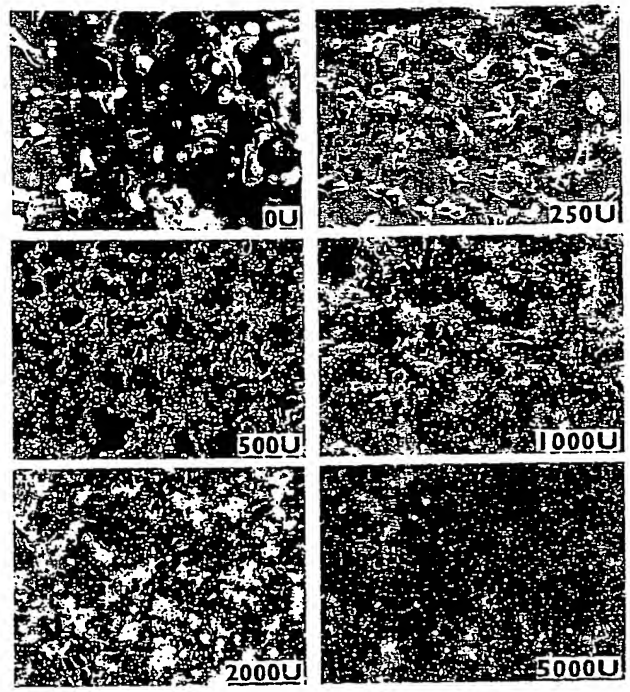

Figure 1. Cytopathic effect in severe acute respiratory syndrome–related coronavirus (SARS CoV)–infected Vero E6 cells. Vero E6 cells were infected with the SARS CoV at an MOI of 0.001 for 1 h and then were incubated in Dulbecco's modified Eagle's medium containing the indicated amounts of interferon–α–2b (IU/mL). Cells were fixed with 4% formalin on day 3 after infection.

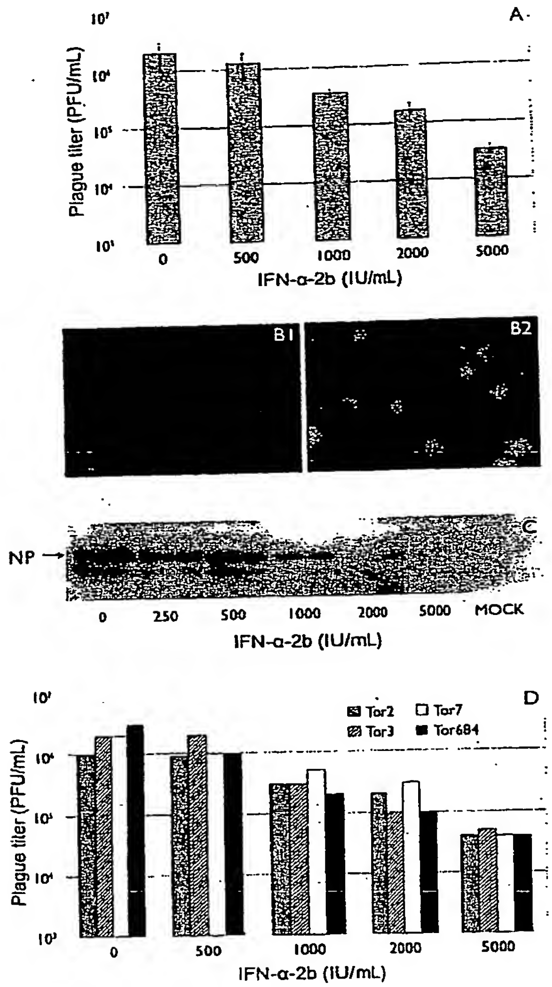

Figure 2. Effect of interferon (IFN)–α–2b on virus growth. *A*, Reduction of virus yield in the presence of antiviral activity. Vero E6 cells were infected with ~100 pfu of the severe acute respiratory syndrome–related coronavirus (SARS CoV). At 1 h after infection, the supernatants were replaced with Dulbecco's modified Eagle's medium (DMEM) or DMEM containing the indicated amounts of IFN-α–2b (IU/mL) for a total of 72 h. Growth of the SARS CoV isolate Tor2 was determined by plaque-titration assay from 3 independent experiments, and data are mean ± SD. *B*, Analysis of cell-to-cell spread of SARS CoV in the presence and absence of IFN-α–2b. Vero E6 cell monolayers were infected with 10-fold dilutions of the SARS CoV. After 1 h of virus adsorption, the inoculum was removed, and cell monolayers were overlaid with 0.9% low-melting-point agarose in DMEM (B2) or DMEM containing 5000 IU/mL of IFN-α–2b (B1). On day 3 after infection, cells were stained with crystal violet. *C*, Protein analysis. Cell lysates were subjected to 10% SDS-PAGE. Protein was electrotransferred to polyvinylidene difluoride membrane (Immobilon P membrane; Millipore). Viral antigen was detected by use of a patient serum sample and horseradish peroxidase–conjugated anti–human IgG, by use of enhanced chemiluminescence. NP, nucleoprotein. *D*, Comparison of the effect of antiviral activity on different SARS CoV isolates. Vero E6 cells were infected with 4 independently isolated SARS CoVs (isolates Tor2, Tor3, Tor7, and Tor684) and were analyzed for their sensitivity to IFN-α–2b, as described above.

the Tor2 isolate (figure 2*D*). For all isolates, the IC_{50} of IFN-α–2b was ~500 IU/mL.

Discussion. The present study has demonstrated that the SARS CoV is not susceptible to ribavirin at high concentrations, even when added at the time of infection. In contrast, the virus is inhibited in tissue culture by IFN-α–2b at concentrations ≥1000 IU/mL. Although the relationship between in vitro and serum IFN concentrations and biological effect is not known, peak serum IFN concentrations of at least 500 IU/mL are observed after intramuscular (im) injection of 3.0×10^7 IU/mL [16]. Similar IFN serum concentrations (100–750 IU/mL) after im injection were reported from a study in patients with hepatitis C virus (HCV)–associated systemic vasculitis [17]. Doses up to 3.6×10^7 IU/mL have been infused intravenously, which might achieve serum concentrations in the range observed to inhibit the SARS CoV. At present, there is no data on the serum levels of IFN-α in patients with SARS, which would be helpful before treatment.

The in vitro studies have thus far only been performed in Vero E6 cells. This is significant, because Vero E6 cells have been shown to have an IFN I gene deficiency and thus are unable to express endogenous IFN I [18]. However, the IFN-dependent pathways are functional and can be activated by exogenously provided IFN. We have tested a limited number of different cell lines (HeLa, 293T, 3T3, and CRFK) for susceptibility to the SARS CoV; thus far, we have failed to identify another line that supports viral replication (data not shown). The observation that the Vero cell clone E6 propagates the virus may indeed be related to the lack of a functional IFN system and, thus, supports the findings in the present study.

Despite efforts by our group and others to grow the virus in different rodent species, a small animal model has not yet been established. Recently, cynomolgus macaques *(Macaca fascicularis)* have been infected with the SARS CoV, which induced similar clinical symptoms and pathological findings to those of humans with SARS [19]. Because only a limited number of animals have been used in the present study, it remains to be determined in the future whether the cynomolgus macaque is a suitable model for SARS and, thus, for antiviral in vivo studies. Until an animal model is definitively established, it might be justified to consider clinical trials with IFN, which is already approved for human use, to determine whether IFN has a beneficial effect on the outcome of SARS CoV infection. Combined with the observation of a lack of efficacy of ribavirin in patients with SARS, our in vitro data further support the conclusion that ribavirin, at least alone, is unlikely to be beneficial in the prophylaxis or treatment of SARS CoV infection. Whether combined therapy with IFN-α–2b and ribavirin would inhibit the replication of the SARS CoV in vitro has not yet been evaluated; the combination is more effective than either agent used alone for the treatment of HCV infection in humans.

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